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The hydrolysis of nicotinamide adenine dinucleotide by brush border membranes of rat intestine

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The hydrolysis of NAD by rat intestine was studied to determine the subcellular site of this hydrolysis and to identify the niacin-containing products that are formed. Using [*nicotinamide*-^{14}C]NAD as substrate, and high pressure liquid chromatography for identification and quantification of products, the present study demonstrates two independent reactions for the hydrolysis of NAD: one that forms nicotinamide through hydrolysis of the ribosyl–pyridinium bond and one that forms nicotinamide mononucleotide through the hydrolysis of the pyrophosphate bond. The nicotinamide mononucleotide is subsequently dephosphorylated to nicotinamide riboside. Enzymes which release nicotinamide mononucleotide and nicotinamide riboside are associated with the brush border membrane as determined by analysis of fractionated intestinal homogenates. The enzyme activity which releases nicotinamide from NAD is associated with the brush border membrane fraction and also with a second cellular particulate fraction. Between pH 5 and pH 6 NAD is hydrolysed principally to nicotinamide. At pH 7.0 rates of nicotinamide and nicotinamide mononucleotide formation are the same. Above pH 7.0 the formation of nicotinamide mononucleotide is preferred.

Most studies of the intestinal absorption of the vitamin niacin (Turner & Hughes, 1962; Spencer & Bow, 1964; Barley *et al.*, 1972; Bechgaard & Jespersen, 1977; Henderson & Gross, 1979; Sadogh-Abasian & Evered, 1980) have been carried out using nicotinic acid or nicotinamide. However, niacin in tissues is largely present in its coenzyme forms. For example, in rat liver, kidney and muscle, niacin content is reflected totally in measurements of nicotinamide nucleotides (Robinson *et al.*, 1947). Therefore, these nucleotides are the major dietary forms of the vitamin niacin; the process by which these coenzymes are handled by the intestine for the purpose of absorption is undefined. Previous studies (Turner & Hughes, 1962) have shown that both the intact rat intestine and intestinal homogenates rapidly hydrolyse NAD to products that were without coenzyme activity and which consisted in part of compounds that had lost the quaternary amine structure.

The present study was undertaken to determine the subcellular site and the properties and products of NAD hydrolysis by rat intestine. A technique employing high pressure liquid chromatography for identification and quantification of the hydrolysis products of NAD is described.

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Materials and methods

[*carbonyl*-^{14}C]NAD (53 mCi/mmol) was purchased from Amersham-Searle. NAD, nicotinamide mononucleotide, nicotinamide, nicotinic acid, nicotinic acid adenine dinucleotide, AMP, adenosine and adenine were purchased from Sigma.

Homogenization and fractionation of mucosal scrapings

Fed Sprague–Dawley rats (200–300 g) were killed by decapitation. The entire small intestine was removed and washed with cold saline and mucosal tissue was scraped off at 4°C. These scrapings were homogenized for 30 s in a Waring blender at 4°C with 15 vol. (w/v) of 0.05 M-mannitol and centrifuged at 10000 *g* for 10 min. The supernatant was decanted and the pellet was resuspended in 5 vol. of 0.05 M-mannitol and rehomogenized for 30 s, then combined with the supernatant, filtered through cheese cloth and a brush border membrane enriched fraction was isolated by the procedure of Schmitz *et al.* (1973). In this procedure the homogenate (H)

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was treated with $CaCl_2$ (final concn. 10mM) and centrifuged twice, once at low speed (2000*g* for 10min) for the removal of a debris pellet (P_1) followed by a high speed (20000*g* for 15min) centrifugation to separate a supernatant (S_1) from a pellet fraction. This latter pellet fraction was further fractionated by resuspension in 0.05M-mannitol (5ml/g of original tissue), rehomogenization with a motor-driven Teflon pestle at 1200rev./min (10 up-and-down strokes), treatment with $MgSO_4$ (10mM final concn.) and two centrifugations, one at 4000*g* for 10min which yielded a second pellet (P_2) and one at 40000*g* for 15min which yielded the final supernatant (S_2) and pellet (P_3). Electron microscopy showed that P_3 is composed of nearly pure brush border membrane vesicles. The various pellet fractions were resuspended in mannitol and analysed for protein (Lowry *et al.*, 1951), for their capacity to hydrolyse NAD (see below) and for the brush border membrane marker maltase (Marx *et al.*, 1972).

For determination of NAD hydrolysis, the protein fraction (0.05–0.15mg) was incubated at room temperature with 2.26mM-[^{14}C]NAD in 25mM-Tris/HCl buffer, pH7.4, containing 2mM-$MgSO_4$. The final volume was 0.50ml. Aliquots (0.05ml) were taken at prescribed times and added to 0.25ml of ice-cold 0.15M-sodium formate buffer, pH3.4. From this mixture a sample (0.05ml) was injected into an anion-exchange high pressure liquid chromatography column (Altex Model 110, Ultrasphere-Si, 5μm, 25cm × 0.46cm internal diameter) which was pre-equilibrated with the same sodium formate buffer. Elution was performed at a rate of 80ml/h and at a pressure of 62–65kg/cm^2. The first 3min consisted of an isocratic elution with the equilibration buffer, the next 4min of a linear gradient (0.15–0.5M) of sodium formate (pH3.4) and the final 3min of 0.5M-sodium formate, pH3.4. The column was regenerated by washing (10min) with the equilibration buffer.

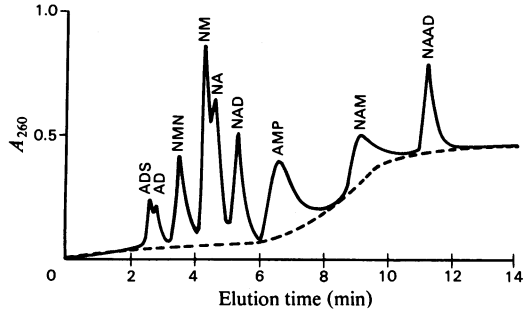

Fig. 1. *High pressure liquid chromatography of potential NAD hydrolysis products*

Abbreviations used: ADS, adenosine; AD, adenine; NMN, nicotinamide mononucleotide; NM, nicotinamide; NA, nicotinic acid; NAD, nicotinamide adenine dinucleotide; AMP, adenosine monophosphate; NAM, nicotinic acid mononucleotide; NAAD, nicotinic acid adenine dinucleotide. The broken line represents the background absorbance of the buffer gradient.

Analysis of the eluate was achieved by continually monitoring the A_{260}, estimation of the individual peak areas by an integrator and the individual collection of the separate peaks for radioactivity measurements. Radioactivity was determined by mixing 0.2ml of the collected sample with Scintisol-complete (Isolab, Akron, OH, U.S.A.) and counting in a liquid-scintillation counter.

Identification of the products was based on the elution time of the standard compounds shown in Fig. 1. Conversion factors to estimate the quantities of substrate hydrolysed as well as the amount of product formed were calculated from individual peak areas obtained after injection of known quantities of test compounds. These determinations were made independently of those based on radioactivity and no major discrepancies were observed between the two determinations.

Results

NAD hydrolysis by homogenates of rat intestine before and after fractionation

Fig. 2 shows the results of high pressure liquid chromatography of [^{14}C]NAD after 1 and 3h incubations with rat intestinal homogenates. The first u.v.-absorbing peak consisted primarily of adenosine; the shoulder of this peak, noted after 3h of incubation, is due to adenine. The second and third peaks consisted of nicotinamide mononucleotide and nicotinamide respectively, and the fourth peak represents unchanged NAD.

Radioactivity determinations of the various peak fractions (see the legend to Fig. 1) revealed the presence of an additional niacin-containing product which co-eluted with adenosine in peak I. This product was found to exhibit strong cationic properties and its chromatographic behaviour, both on paper (Sakai *et al.*, 1972) and column (Bernofsky, 1980) was identical with that of nicotinamide riboside.

These products were the only ones observed during the hydrolysis of NAD by intestinal homogenates. The sum of the amounts of these products, whether determined on the basis of u.v. absorption or by radioactivity measurements, represented the total net loss of NAD. Recovery from the column was greater than 95%.

The hydrolysis of NAD is catalysed principally by enzymes in the particulate fractions of the intestinal homogenates as shown in Table 1. The

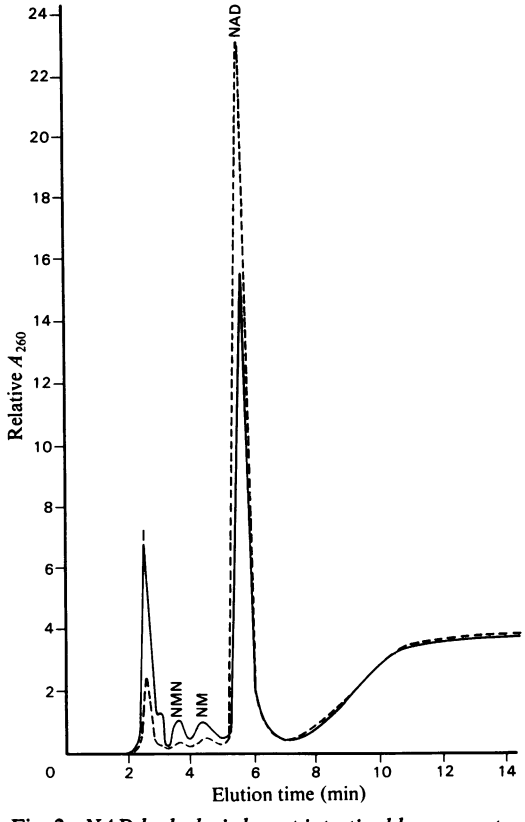

Fig. 2. *NAD hydrolysis by rat intestinal homogenates*
The intestinal homogenate (0.15 mg of protein) was incubated with 2.26 mM-[^{14}C]NAD at pH 7.4 as described in the Materials and methods section. Samples were withdrawn and analysed for hydrolysis products by high pressure liquid chromatography. The broken line represents the chromatogram after 1 h incubation; the solid line represents the chromatogram after 3 h incubation. The amount of radioactivity (nmol) in each peak after 1 and 3 h respectively was: peak I, 0.14, 0.70; NMN peak, 1.19, 3.74; NM peak, 1.17, 2.72; NAD peak, 17.30, 12.60.

formation of nicotinamide mononucleotide and nicotinamide riboside are the property of the brush border membrane. Both activities were enriched about 11-fold in the purified brush border membrane fraction, P_3, which also contained 33% of each activity compared with the original homogenate.

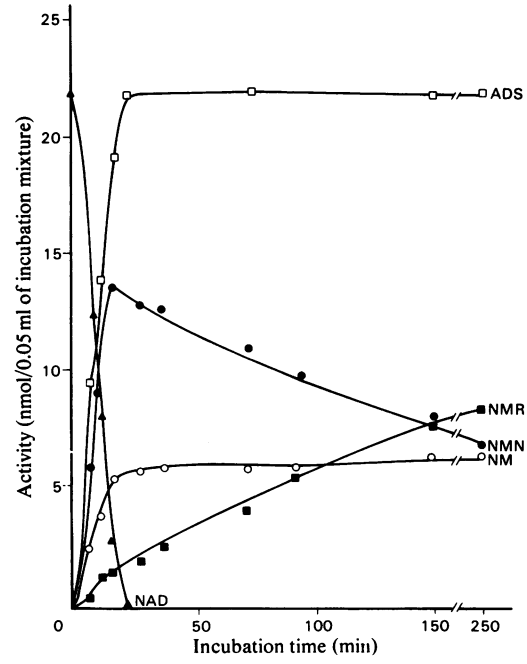

Fig. 3. *Time-dependence of NAD hydrolysis by brush border membrane fraction*
The membrane fraction preparation (0.05 mg of protein) was incubated at room temperature and pH 7.4 with [^{14}C]NAD. Aliquots (0.05 ml) were withdrawn at the indicated times and analysed by high pressure liquid chromatography. Abbreviations are as given in the legend to Fig. 1, except that NMR stands for nicotinamide riboside [value based on radioactivity in the adenosine peak (see Fig. 2)].

Table 1. *Hydrolysis of NAD by rat intestinal homogenates before and after fractionation*
All activities are nmol/min per mg of protein, except for maltase (μmol/min per mg of protein). Values in parentheses are % of total.

Fraction	Protein (mg)	Specific activity of		Formation of		
		Maltase	NAD hydrolysis	Nicotinamide riboside	Nicotinamide mononucleotide	Nicotinamide
Homogenate	516	0.4	16.6	1.0	9.9	4.7
P_1	198	0.3 (28)	15.7 (36)	0.7 (26)	7.1 (27)	6.6 (54)
S_1	209	0.12 (12)	6.3 (15)	0.3 (14)	4.0 (17)	1.2 (11)
P_2	6	3.9 (11)	142 (10)	9.5 (11)	92 (11)	38.6 (10)
S_2	3.8	1.3 (2)	33.3 (2)	4.2 (0.4)	32 (0.3)	0
P_3	14.5	4.7 (3.2)	160 (27)	11.4 (33)	118 (33)	25.5 (15)
Enrichment (P_3/homogenate)		11.7	9.6	11.4	11.9	5.4

This pattern of distribution and enrichment is the same as that of the brush border membrane marker maltase. The formation of nicotinamide is different in some respects; although it is also concentrated in the particulate fractions, the purified brush border membrane fraction, P_3, contains only 15% of the activity and is only 5.4-fold enriched compared with the original homogenate.

Time dependence of NAD hydrolysis in the brush border membrane fraction

The time dependence of NAD hydrolysis and the appearance by purified brush border membranes (P_3) at pH 7.4 of products is described in Fig. 3. The disappearance of NAD from the reaction mixture was rapid, with a rate constant of $0.2\,min^{-1}$, and was complete after 20 min of incubation. This coincides with the appearance of adenosine, nicotinamide mononucleotide and nicotinamide at molar proportions of about 1:0.7:0.3, respectively. After the exhaustion of NAD the amounts of adenosine and nicotinamide remained unaltered while the amount of nicotinamide mononucleotide declined. The appearance of nicotinamide riboside occurred after a lag period of about 5 min and the amount increased continuously until the supplies of nicotinamide mononucleotide were exhausted.

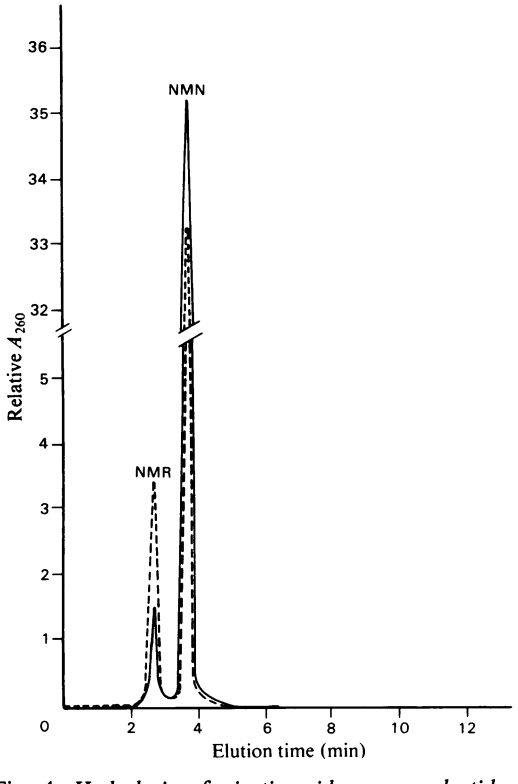

Fig. 4. *Hydrolysis of nicotinamide mononucleotide by brush border*
Incubation conditions were as described in Fig. 3 except that nicotinamide mononucleotide was added instead of NAD. The solid line is the chromatogram after 30 min incubation; the broken line is the chromatogram after 60 min incubation.

Hydrolysis of nicotinamide mononucleotide

The high pressure liquid chromatograph in Fig. 4 shows the results when nicotinamide mononucleotide is directly exposed to brush border membranes at pH 7.4. After 7 h incubation, nicotinamide riboside was the only product formed from nicotinamide mononucleotide. No nicotinamide formation was detected from either nicotinamide mononucleotide or nicotinamide riboside under these incubation conditions.

Incubation of the brush border membrane fraction with nicotinamide (results not shown) did not result in the formation of any other products (e.g. nicotinic acid).

pH dependence of NAD hydrolysis by brush border membrane

The studies presented in Fig. 5 show the formation of NAD hydrolysis products as a function of

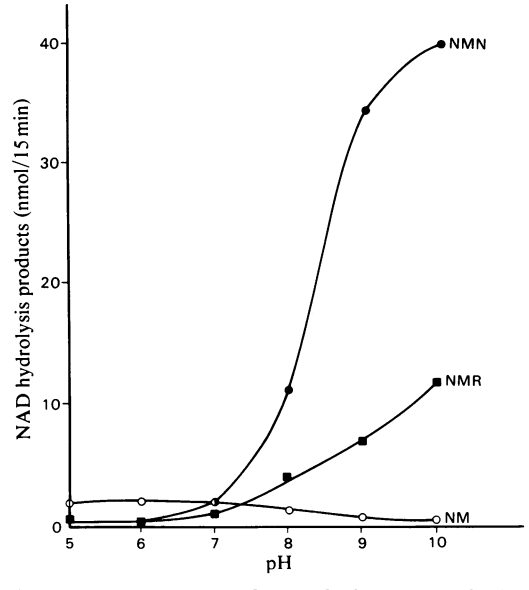

Fig. 5. *pH dependence of NAD hydrolysis by the brush border membrane preparation*
The membrane preparation (0.05 mg of protein) was incubated for 15 min at room temperature with [^{14}C]NAD in 25 mM-buffered salt solutions of the indicated pH containing 2 mM-$MgSO_4$. Buffered salt solutions were made of acetic acid adjusted to the desired pH with 1 M-Tris or -NaOH.

pH. The formation of nicotinamide had a broad pH optimum extending between pH 5.0 and pH 7.0. This activity decreased gradually with increasing pH. The formation of nicotinamide mononucleotide and nicotinamide riboside was negligible at pH values below 6.0 but became comparable with that of nicotinamide at pH 7.0, and then rose sharply with an increase in pH.

Discussion

The present study shows that a homogenate of rat intestine hydrolyses NAD to nicotinamide, nicotinamide mononucleotide and nicotinamide riboside. Turner & Hughes (1962), basing their assays on loss of NAD coenzyme activity and loss of quaternary amine structure, suggested the formation of two major products and were unable to detect nicotinamide riboside. Furthermore they suggested the formation of some nicotinic acid. We were unable to detect any significant formation of nicotinic acid either by the crude homogenates or by any of the fractions tested. This raises the possibility that the nicotinic acid detected by Turner & Hughes (1962) could have arisen from the action of bacterial contaminants of the intestinal preparations (Chiang *et al.*, 1972; Henderson & Gross, 1979).

Both the nicotinamide riboside-forming and nicotinamide mononucleotide-forming activities appear to be solely associated with the brush border membranes (Table 1). The enrichment and distribution of these two activities in the various fractions of the intestinal homogenate closely resemble those of the brush border enzyme maltase. The nicotinamide-forming activity, on the other hand, appears to be the property of both the brush border membrane and another subcellular particulate fraction. This is suggested by the finding that a greater percentage of the nicotinamide-forming activity was found in the first pellet fraction (P_1) while the enrichment in the brush border membrane fraction is half of that for maltase. No major contamination with other cellular markers (Selhub & Rosenberg, 1978) was observed in this membrane fraction.

The time-dependence studies conducted with the brush border membranes indicate the presence of at least two separate enzymes, one that forms nicotinamide through the hydrolysis of the ribosyl–pyridinium bond and an NAD pyrophosphatase, which are directly involved in the hydrolysis of NAD. As shown in Fig. 3, nicotinamide formation occurs without an initial lag period and ends when the NAD substrate is exhausted. No nicotinamide is formed from nicotinamide mononucleotide under these conditions (Figs. 3 and 4). On the other hand, nicotinamide riboside appears to be formed from the mononucleotide derivative, because the formation of the riboside occurs after an initial lag period, and after exhaustion of the NAD substrate the riboside continues to be formed in a manner proportional to nicotinamide mononucleotide disappearance.

The physiological significance of these findings awaits further studies to determine if ingested niacin coenzymes are hydrolysed by other enzymes and by different mechanisms than those described here. Thus far we have found no measurable NAD hydrolytic activity in rat pancreatic or biliary fluid. If other less likely sources, such as saliva and gastric juice, are also devoid of these activities it would suggest that niacin coenzyme degradation takes place largely as a function of intestinal brush border enzymes. Such hydrolysis apparently precedes the uptake of nicotinamide by intestine. The fate of nicotinamide mononucleotide, nicotinamide riboside and NAD with regard to intestinal absorption is yet to be determined.

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